IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Jan Zavada et al		
Serial No. :	08/260,190	Group:	1800
Filed :	June 15, 1994	Group Art Unit:	1804
For :	MN Gene and Protein	Examiner:	D. Curtis Hogue, Jr.

DECLARATION UNDER 37 CFR SECTION 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DIETER COTTER GRUENERT hereby declares and states:

1. I received a Ph.D. degree in Radiation Biophysics in 1982 from the University of California, Berkeley. In an intercampus program in Biophysics between the University of California, San Francisco, and University of California, Berkeley, I performed research on DNA repair mechanisms at the Laboratory of Radiobiology and Environmental Health, University of California, San Francisco. I was a post-doctoral fellow in the Department of Carcinogenesis, Institut Suisse de Recherches Experimentales sur le Cancer, from 1982 to 1984, where I performed research on regulation of

Appendix I

oncogene expression by oxidizing agents and cloning of the gene for Fanconi's anemia. From 1984 to 1986, I was a Molecular Biologist at Hana Biologics, Inc., Berkeley, CA.

2. From 1986 to the present, I have been at the University of California, San Francisco, California, where I currently am a member of the Program in Biological Sciences and a member of the Graduate Program in Biomedical Sciences. Also, I am a Member of the Graduate Group in Oral Biology, a Member of the Graduate Group in Biophysics, an Associate Member of the Cardiovascular Research Institute, an Associate Professor in Residence in the Department of Laboratory Medicine, an Adjunct Associate Professor in the Department of Stomatology, and the Co-Director of the UCSF Gene Therapy Core Center. I have presented more than 80 invited lectures in the fields of cellular transformation including roles of oncogenes. Additionally, I have published over 60 peer-reviewed articles in the fields of gene therapy including antisense therapy, homologous recombination, cellular transformation, gene expression, roles of oncogenes in neoplastic progression, development of expression systems to study gene function, including antisense suppression of gene transcription and translation. These publications include Wagner et al. "Antisense oligodeoxynucleotides to the cystic fibrosis transmembrane regulator inhibit cAMP-activated but not calcium activated chloride currents", Proc. Natl. Acad. Sci. USA, 89:6785-6789, 1992. I have published 16 book chapters, review articles and editorials in the fields of cellular transformation and DNA repair, including 4 publications in

gene-targeting and gene therapy. Finally, I have published over 90 abstracts in the above fields.

3. I have read the relevant portions of the Specification entitled MN Gene and Protein, Serial No.: 08/260,190 filed June 15, 1994 and the claims currently under consideration.

(a) I declare that the in vitro results shown in the subject specification, for example at pages 65 - 67, reasonably predict in vivo therapeutic efficacy of MN antisense oligonucleotides for the following reasons. First, there is a strong association of MN gene expression with tumorigenesis. Second, transfection experiments with MN sense and antisense constructs, in non-tumorigenic and tumorigenic cell lines, respectively, show that MN sense constructs cause non-tumorigenic cells to exhibit a transformed phenotype, whereas the antisense constructs cause the tumorigenic cells to have a very much lowered proliferation rate and to form smaller colonies than controls. Third, prior studies show that the in vitro effects observed in studies of other, structurally similar oligonucleotides, correlate with in vivo therapeutic effects. I will describe each of these points in more detail in sections 4 - 6 below.

(b) I also declare that the in vitro screening studies shown would permit workers of skill in the art to select antisense MN oligonucleotides which would be therapeutically useful. I will describe each of these points in more detail in sections 7 and 8 below.

4. The Specification discloses the close association of MN gene expression and human cancer. Example 6, pages 109 - 111, Figure 9, described at pages 110 - 111, and Example 13, described at pages 123 - 132, present immunological studies which indicate that with the exception of the normal gastrointestinal epithelium, MN is expressed to a significant extent only in human cancer cells. This data is summarized in Table 3 at page 131.

5. The Specification at pages 65 - 67, and at pages 133 - 137 describes the relationship between MN expression and cell growth.

(a) The Specification at page 65 reports that, compared to control cells, transfection with a plasmid containing MN cDNA increased the proliferation rate and plating efficiency of CGL1 cells, a human non-tumorogenic cell line.

(b) A similar type of experiment using NIH 3T3 cells is reported in Example 15, described at pages 133 - 137. This cell line was appropriately chosen because of its prior successful use in studies of phenotypic expression of a number of proto-oncogenes. Findings described at page 136 showed that compared to control cells, MN-expressing 3T3 cells displayed spindle-shaped morphology and increased refractility, they were less adherent to the solid support, and they were smaller in size. Furthermore, the MN expressing cells lost the capacity for growth arrest and grew chaotically on top of one another (Figure 23 a-d). Moreover, MN-transfected 3T3 cells grew in soft agar. The data presented in Table 3 at page 136 show that transfection with a plasmid containing MN cDNA decreased

the doubling time, increased saturation density, and increased cloning efficiency of 3T3 cells compared to cells transfected with the pSG5C plasmid not containing the MN cDNA. In contrast, the control 3T3 cells had flat morphology similar to parental NIH 3T3 cells, were aligned with each other, thus forming an ordered monolayer. Thus, those studies showed that transfection with a plasmid containing MN cDNA confers tumorigenic properties upon a previously non-tumorigenic cell line.

(c) Conversely, the specification at pages 65 - 67 described studies in which a plasmid containing an antisense MN cDNA had an opposite effect in a tumorigenic cell line, CGL3. Compared to non-transfected cells, the antisense-transfected tumorigenic cells formed smaller colonies and had a very much lower proliferation rate. Thus, I declare that the results from the transfection experiments reported in the specification with sense and antisense nucleic acids in non-tumorigenic and tumorigenic cell lines shows that MN expression affects cell growth and is strongly correlated with tumorigenesis.

(d) The studies of CGL1 cells, 3T3 cells, and CGL3 cells each support the contention that MN is an oncogene that can regulate the abnormal growth of cancer cells. Thus, I declare that the above-described studies, in combination with the immunological studies described above in paragraph 4 provide a strong basis for expecting that inhibiting MN expression using antisense oligonucleotides would inhibit the growth of MN expressing tumor cells.

6. I declare that I am aware of the published literature at the time of filing the application, and that this literature generally taught the relationship between antisense oligonucleotide structure and efficacy of in vivo inhibition of the expression of any gene whose DNA sequence is known. [See for example, Zamecnick, P.D., Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, Wiley-Liss, Inc., New York, NY, USA; (1991), pages 1 - 6; cited on page 92 of the Specification.] The work of Mirabelli et al., Anti-Cancer Drug Design, 6:647-661 (1991), described some of the studies which demonstrated efficacy of antisense ODNs structurally similar to those of the present invention in inhibiting transcription of the oncogenes, c-myc, c-myb, BCL-2, and N-ras. Further, studies of Kitajima et al., Science 258:1792-1795 (1992), Ratajczak et al., Proc. Nat. Acad. Sci. 89:11823-11827 (1992), Chiasson et al., Eur. J. Pharm.- Mol. Pharm. Section 227:451-453 (1992) and Wickstrom et al., Cancer Res. 52:6741-6745 (1992) each support the use of antisense ODNs of similar structure to MN antisense ODNs in treating diseases in vivo. Zamecnick, Ratajczak et al., Chiasson et al., and Wickstrom et al. were cited in the Specification or in the Information Disclosure Statement filed for this application. A copy of Mirabelli et al. is appended to this declaration. Because of the strong correlation between MN expression and tumorigenesis, and because of the structural similarity between MN antisense oligonucleotides and oligonucleotides shown in the above-identified references to inhibit expression of other cancer genes and cellular proliferation in vivo, I declare that the in vitro inhibition of cellular growth by MN antisense nucleic acids

as shown at pages 65 - 67 is reasonably predictive of the efficacy of MN antisense oligonucleotides in treating MN-expressing human cancer.

7. The Specification also described a screening procedure using HeLa cells in vitro for selecting therapeutically useful antisense MN oligonucleotides. Example 10, described at pages 118 - 120, and Figure 3, show that MN antisense oligonucleotides suppressed MN gene expression in those human cancer cells. I am aware that the Specification contains a typographical/proofreading error at page 119 line 25, erroneously stating that there was an "increase" in MN expression with the combination of ODN1 and ODN2 together. However, I declare that Figure 3C shows a decrease in MN expression, not an increase.

8. I further declare that the published literature taught routine methods for designing, making, delivering, and evaluating oligonucleotides using for successful in vivo use. Thus, I declare that I believe that a worker of ordinary skill would be able to use the routine screening system described in Example 10 to select therapeutically useful MN antisense oligonucleotides. Based on the studies reported in the specification, it can be reasonably assumed that an MN antisense oligonucleotide which inhibits expression of MN protein in MN expressing tumorigenic cell lines, such as HeLa or CGL3 cell lines, would be therapeutically useful.

9. I additionally declare that the disclosure of the M75 monoclonal antibody in the specification, and the deposit of the cell line VU-M75 under the Budapest Treaty at the American Type Culture Collection (ATCC) on

September 17, 1992 under ATCC No. NB 11128 would have permitted a worker of ordinary skill in the art to determine the correct sequence of the MN cDNA and to design antisense oligonucleotides for therapeutic use. The type of sequencing errors apparent from the original sequencing data, namely GC compressions, are not uncommon in the art, and are easily corrected, given the disclosure of a specific monoclonal antibody, such as M75.

10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

May 28, 1997
Date

Dieter Cotter Gruenert, Ph.D.

Anti-Cancer Drug Design (1991), 6, 647–661

In vitro and *in vivo* pharmacologic activities of antisense oligonucleotides

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Summary: The use of antisense oligonucleotide as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and chemical properties that govern the structure of nucleic acids. Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous ocogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. The therapeutic activity of antisense compounds in animal disease models have also been reported.

This review provides some general conclusions and trends regarding the pharmacologic action of antisense oligonucleotides, that can be formulated from studies previously reported in the literature. In addition, data is highlighted for two specific examples in which antisense oligonucleotides have demonstrated activity against herpes viruses and intracellular adhesion molecule RNA targets.

Introduction

In the past few years, many papers have been published demonstrating the activity of numerous antisense oligonucleotides, of different sequences and chemical type, in a variety of cell-based systems. Recently there have been a number of excellent reviews that have summarized the activities of these compounds in detail (Cohn, 1989; Uhlman & Peyman, 1990; Cazenave & Helene, 1991). As such this review will not attempt to duplicate those comprehensive efforts; instead it will provide a brief summary of the activities of oligonucleotides in cell-based assays and attempt to provide some general conclusions and trends that can be formulated from these previously published data. In addition, this paper will provide examples of data compiled in our laboratories that relate to the pharmacological activities of phosphorothioate oligonucleotides directed against cellular and infectious disease targets.

Pharmacological activities in cell-based models

Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous oncogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. These studies varied in the types of oligonucleotides used, the cells used, the RNAs and specific receptor sequences targeted and the conditions employed. Although a wide range of oligonucleotide concentrations have been used to treat cells, only a few studies have reported detailed dose-response curves and clearly documented the purity of the oligonucleotides used. Table I summarizes the information from more than 40 papers in which oligonucleotides were tested for pharmacologic

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Table I Summary of cell-based *in vitro* activities of antisense oligonucleotides

Target viruses	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	Zamecnik *et al.* [1986]
HIV	H-T cells	+	P-S	14–28	0.5 μM	Matsukura *et al.* [1987]
HIV (gag/pol)	H-T cells	+	P-S	18–24	1–10 μM	Kinchington & Galpin [1989]
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	Agrawal *et al.* [1988]
HIV	CZM cells	+	PS	18–28	10 μM	Vickers *et al.* [1991]
Herpes simplex	Vero cells	+	CH3-P	7	50–100 μM	Smith *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	Gao *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P	12	20–50 μM	Kulka *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P-psoralen	12	5 μM	Kulka *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	Draper & Brown-Driver [1991]
Vesicular stomatitis	L929 cells	+	CH3-P	9	25–50 μM	Agris *et al.* [1986]
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	Shea *et al.* [1991]
Vesicular stomatitis	L929 cells	+	P-poly l-lysine	10–15	0.1 μM	LeMaitc *et al.* [1987]
Influenza	MDCK cells	+	P-acridine	11	50 μM	Zerial *et al.* [1987]
Tick borne encephalitis	–	+	Various	Various	0.1–1 μM	Vlassov [1989]
SV40	MDCK cells	+	CH3-P	6–9	25 μM	Miller *et al.* [1985]
Rous	Chicken fibroblasts	+	Various	Various	10 μM	Zamecnik & Stephenson [1978]
Hepatitis B	Alexander	+	P	15	8.5 μM	Goodarzi *et al.* [1990]
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	Cowsert & Fox [1991]

Herpes simplex	Hela cells					[1991]
Vesicular stomatitis	L929 cells	+	CH3-P	9	25–50 μM	Agris *et al.* [1986]
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	Shea *et al.* [1991]
Vesicular stomatitis	L929 cells	+	p-poly l-lysine	10–15	0.1 μM	LeMaite *et al.* [1987]
Influenza	MDCK cells	+	P-acridine	11	50 μM	Zerial *et al.* [1987]
Tick borne encephalitis	—	+	Various	Various	0.1–1 μM	Vlassov [1989]
SV40	MDCK cells	+	CH3-P	6–9	25 μM	Miller *et al.* [1985]
Rous	Chicken fibroblasts	+	Various	Various	10 μM	Zamecnik & Stephenson [1978]
Hepatitis B	Alexander	+	P	15	8.5 μM	Goodarzi *et al.* [1990]
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	Cowsert & Fox [1991]
Oncogenes						
c-myc	T-lymphocytes	+	P	15	30 μM	Heikkila *et al.* [1987]
c-myc	HL-60 cells	+	P, PS	15	10 μM	Wickstrom *et al.* [1989]
c-myc	Burkitt cells	–	P	21	100 μM	McMananway *et al.* [1990]
c-myb	PMBC	+	P	18	40 mg/ml	Gewirtz *et al.* [1989]
BCL-2	L697 cells	–	P ± PS	20	25–150 μM	Reed *et al.* [1990]
N-ras	T15 cells	+	CH3-P	9	Inactive	Tidd *et al.* [1988]
Host genes						
Multiple drug resistance	MCF-1 cells	+	PS	15	—	Jaroszewski *et al.* [1990]
PCNA (cyclin)	3T3	+	P	18	30 μM	Jaskulski *et al.* [1989]
Prothymosin	Human myeloma cells	–	P	16–21	40 μM	Sburlati *et al.* [1991]
T cell receptor	T cells	+	P	22	—	Zheng *et al.* [1989]
Gm CSF	Endothelial cells	–	P	15, 18	10^{-5} M	Zheng *et al.* [1989]
CSF-1	FL-ras/myc cells	+	P	—	—	Birchenall *et al.* [1989]
EGF receptor		+	P	13	30 μM	Yeoman *et al.* [1989]
β Globin	Rabbit reticulocytes	+	CH3P	9	100 μM	Blake *et al.* [1985]
cAMP-protein kinase II_β	HL-60	+	P	21	15 μM	Tortora *et al.* [1990]
Phospholipase A2 Activating protein	BC3H1	+	P	25	25 μM	Clark *et al.* [1991]
bFGF	Human astrocytes	–	P	15	10–75 μM	Morrison [1991]
TAU	Neurons	–	P	20–25	3–50 μM	Caceres & Kosik [1990]
Myeloblastin	HL-60 cells	+	P	18	—	Bories *et al.* [1989]
ICAM-1	A549, HUVEC	–	PS	18–20	0.01–1 μM	Chiang *et al.* [1991]
IL-2	T lymphocytes	–	P	15	5 μM	Harel-Bellan *et al.* [1988]
IL-1α	HUVEC	+	P	18	10 μM	Maier *et al.* [1990]
IL-1β	Monocytes	+	PS	15	0.1–2.5 μM	Manson *et al.* [1990]
IGF-1	Myoblasts	–	P	15	10 μM	Florini & Ewton [1990]
Perforin	T lymphocytes	–	P	18	5–35 μM	Acha-Orbea *et al.* [1990]

Table I (*contd.*)

Target	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH3P	21	5–30 μM	Marcus-Sekura *et al.* [1987]
Placental alkaline Phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18–28	0.25–5 μM	Vickers *et al.* [1991]
Chloramphenicol acetyl transferase driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	Cowsert & Fox [1991]

cAMP = cyclic AMP; EGF = epidermal growth factor; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor; HB = hepatitis B; HIV = human immunodeficiency virus; HSV = herpes simplex virus; HTLV = human T cell lymphotrophic virus; IV = influenza virus; PCNA = proliferating cell nuclear antigen; RSV = Rous sarcoma virus; TAR = TAT response element; TBE = tick-borne encephalitis; VSV = vesicular stomatitis

activities against a variety of viruses, oncogenes, host genes and transfected reporter genes.

The data presented in Table I support only a few generalizations. First, while phosphodiesters are rapidly degraded in biological systems, a number of investigators have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum or in medium supplemented with heat-inactivated serum. When phosphodiester oligonucleotides have displayed activity, concentrations of more than 10 μM were required. The explanation for these activities is unclear. Considering the presence of endo- and exonucleases that are found within cells it is reasonable to think that these oligonucleotides would be degraded in the cell very rapidly. Evidence from our laboratory demonstrates that in a number of routinely used cell lines phosphodiester oligonucleotides are degraded within minutes by nucleases found in the plasma membrane, cytoplasm and in nuclei (Hoke *et al.*, in press).

Second, a variety of chemically modified oligonucleotides have been reported to be active in cell culture. Although considerable variation has been reported, phosphorothioate oligonucleotides appear to be more potent than methylphosphonate oligonucleotides. Conjugation of alkylators and interchelators to phosphodiesters and methylphosphonates has been reported to increase potency. Many of these modifications have been positioned at either the 3′ or 5′ end of the oligonucleotides; 3′ positioning is an attempt to increase stability to 3′-exonuclease, the predominant serum nuclease. Lipophilic and poly(L-lysine) conjugates have also displayed enhanced potencies presumably via some modulation of cellular pharmacokinetic characteristics.

Third, oligonucleotides have demonstrated activities against a broad array of viral targets, oncogenes, normal cellular gene products and various transfected genes. This array of pharmacological effects clearly demonstrates the broad potential therapeutic applicability of these drugs.

Fourth, although the data from studies included in Table I are limited, when it is combined with *in vitro* toxicologic data (Crooke, 1991), the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high. Initial data regarding certain phosphorothioates of 20 and 21 nucleosides in length, targeted to human papilloma virus and herpes simplex virus, respectively, also demonstrate that these compounds are extremely well tolerated in animals (Mirabelli *et al.*, in preparation). The effects of specific base composition within an oligonucleotide, oligonucleotide length, specific chemical modifications in oligonucleotide and cellular parameters (i.e. cell type, cell cycle phase and stages of differentiation) on the potential toxicology and non-antisense activities of these compounds are not yet clearly defined (Crooke, 1991).

Fifth, very little data that support putative mechanisms of action have been reported and generalizations concerning precise mechanisms of action are not possible. A variety of mechanisms have been proposed to explain the ultimate pharmacologic action of antisense oligonucleotides, all resulting from the hybridization of the drug with the complementary sequence within a target RNA. These mechanisms include the disruption of ribosomal assembly and function, formation of an RNase H substrate and subsequent cleavage of the target RNA, and disruption of RNA splicing processes or other RNA metabolic processes. It is very likely that many 'terminating' mechanisms can be exploited for the cellular action of antisense oligonucleotides and that the mechanisms of a particular oligonucleotide are the result of the particular RNA and sequence target, the cell in which the drug is acting and the chemical structure of the oligonucleotides.

Examples of antisense pharmacologic activities

Our laboratory has demonstrated activities of oligonucleotide drugs against a number of molecular disease targets. Below is a brief summary of work on two targets: herpes simplex virus and a human cell adhesion molecule, ICAM-1. These data are reviewed in an attempt to provide examples of the antisense drug discovery process and the activities of antisense compounds directed against viral gene targets and host gene targets.

Antisense oligonucleotides directed to herpes simplex virus RNA targets

In vitro activities. Smith & Smith (1986) first reported antisense inhibition of HSV replication using oligonucleotides targeted to the splice junction sequences of the HSV-1 1E4 and 1E5 pre-RNAs. It was later reported that increasing the length of the oligonucleotide increased the antiviral activity against HSV-1 (Kulka *et al.*, 1989). The most active oligonucleotide, a 12-nucleotide long oligomethylphosphonate, was directed against a splice junction covering six nucleotides in both exon and intron. The potency of the compound was greatest when added at the time of infection (IC_{50} = 15 μM) with a 5- to 10-fold reduction in potency when the oligonucleotide was added 1 h post-infection. A 20% inhibition in splicing was observed in oligonucleotide treated infected cells *versus* untreated infected cells. Conjugation of the 12-mer oligomethylphosphonate with a psoralen-derivative increased the potency of the compound approximately 3-fold relative to the unconjugated compound. However, the psoralen conjugate required activation by UV irradiation following addition to the infected cells.

A study by Draper *et al.* (1990) using phosphodiester oligonucleotides complementary to two related region of the HSV-1 Vmw 65 mRNA, reported that an oligonucleotide targeted to the translation initiation region effectively inhibited HSV-1 replication. The other oligonucleotide was inactive, causing these authors to conclude that sequences within the same mRNA can exhibit differential sensitivities to antisense oligonucleotides.

Our laboratory has designed and tested several oligonucleotides which are complementary to the translation initiation regions of several mRNAs of HSV. Oligonucleotides which target the HSV UL13 mRNA were found to be effective inhibitors of HSV replication, as measured in an infectious yield assay (Draper & Brown-Driver, 1991; Draper *et al.*, submitted). The protein encoded by the UL13 gene has been putatively identified as a phosphotransferase which may be involved in the phosphorylation of viral capsid proteins (Smith *et al.*, 1986; Stevely *et al.*, 1985). Preliminary screening experiments revealed that phosphorothioate oligonucleotides were significantly more potent than phosphodiester and methylphosphonate oligonucleotides (Draper & Brown-Driver, 1991; Draper *et al.*, submitted). One of the most potent compounds evaluated was ISIS 1082, a 21-mer phosphorothioate oligonucleotide, targeted to a secondary initiation codon present in HSV-1 and HSV-2 UL13 mRNA. This compound inhibited both HSV-1 (KOS strain) and HSV-2 (HG52 strain) replication in an infectious yield assay. Site specific cleavage of synthetic UL13 transcripts was induced by addition of ISIS 1082 in RNA processing extracts of HeLa cells suggesting that ISIS 1082 may inhibit expression of the UL13 gene product by inducing RNAase H specific cleavage of UL13 mRNA.

Evaluation of the compound in infectious yield assays using acyclovir sensitive and resistant strains and in comparative dose responses with acyclovir and other phos-

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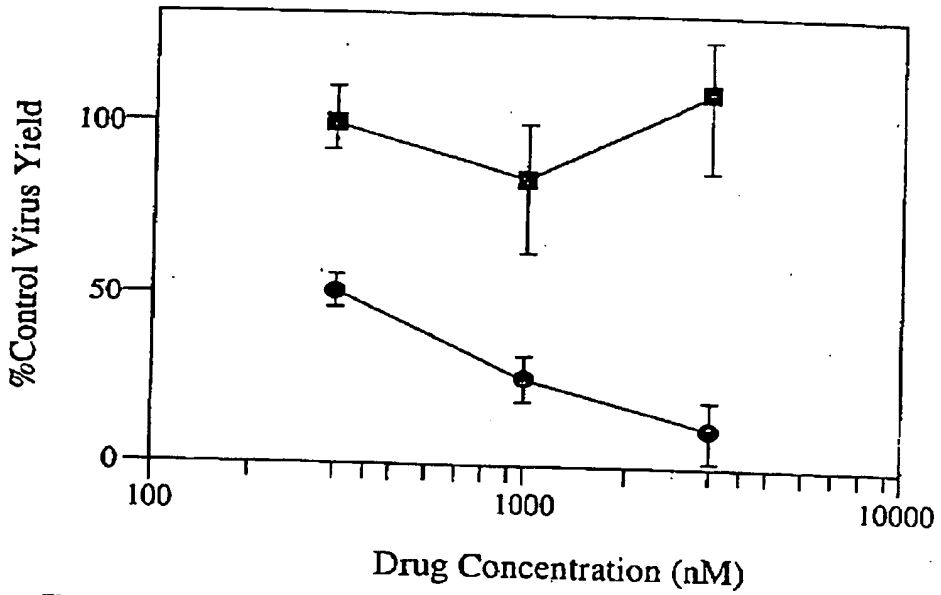

Figure 1 Sensitivity of an acyclovir resistant strain of HSV-1 (DM 2.1, thymidine kinase deletion mutant) to ISIS 1082 (●) and acyclovir (■). Activities were measured in an infectious yield assay and expressed as a percent of untreated infected cell virus yield

phorothioate oligonucleotides provided more evidence that ISIS 1082 produces its antiviral activity via a sequence specific antisense effect. First, ISIS 1082 inhibits the replication of the HSV-1 strain KOS in HeLa cells by 50% and 90% at concentrations of approximately 300 nM and 2 μM, respectively. ISIS 1082 was 3- to 10-fold more potent then a phosphorothioate oligonucleotide of similar length and equivalent (but scrambled) nucleotide base composition when tested against certain strains of HSV-1 and HSV-2. In addition, it was found that ISIS 1082 was active against a number of acyclovir resistant strains of HSV-1. Figure 1 shows the activity of ISIS 1082 against the thymidine kinase deletion mutant strain, DM2.1. Acyclovir did not inhibit the replication of this strain. However, treatment with ISIS 1082 resulted in a dose-dependent decrease in infectious viral yield that was equivalent to that observed against the KOS strain of HSV-1. At concentrations as high as 100 μM of ISIS 1082, only minimal effects on host cell growth and metabolism were observed (Crooke *et al.*). This lack of *in vitro* toxicity is again consistent with the postulated highly selective mode of action of the antisense compound.

In vivo activities. Earlier preliminary reports have suggested *in vivo* activities of antisense drugs against HSV infections. One report indicated that an oligomethylphosphonate was active in a mouse model of herpes simplex virus 1 infection (Kulka *et al.*, 1989). Two additional laboratories have reported on the activity of phosphorothioates against HSV-1 infections in mouse models of ocular herpetic keratitis (Kimura *et al.*, 1991; Metcalf *et al.*, 1991).

Recent data have demonstrated that topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with HSV-1 (KOS) resulted in curative activity at drug concentrations of 0.3% and 5% (Brandt *et al.*, 1991; Brandt *et al.* submitted). The activity of ISIS 1082 in this model was equivalent to trifluorothymidine and exhibited no local or systemic toxicities. ISIS 1082 is currently being studied in rabbit models of HSV-1 induced epithelial keratitis and other animal models of dermal and systemic HSV infection to better define the pharmacology of the compound.

Antisense oligonucleotides directed to intercellular adhesion molecules

To date most reports of antisense oligonucleotide activities in non-viral infection models have focused on oncogene targets and receptor signaling targets as seen in Table I. Our laboratory has recently explored the use of antisense oligonucleotides to pharmacologically manipulate the expression of certain cellular adhesion molecules (Chiang *et al.*, 1991).

Rationale for adhesion molecules as antisense targets

The binding of circulating leukocytes to vascular endothelium is an obligatory step in the emigration of leukocytes out of the vasculature to the site of infection or injury (Harlan, 1985). Several endothelial proteins have recently been identified which mediate the adherence of leukocytes to inflamed vascular endothelium and subsequent migration out of the vasculature (Stoolman, 1989; Osborn, 1990; Springer, 1990). One such protein, ICAM-1, is a 95–105 kD glycoprotein first identified by the ability of a monoclonal antibody to block phorbol ester induced aggregation of a B-cell line (Rothlein *et al.*, 1988). The cellular distribution of ICAM-1 is different from other endothelial cell adhesion molecules in that it is expressed in both endothelial cell and non-endothelial cells including leukocytes, fibroblasts, kernatinocytes and other epithelial cells (Table II). ICAM-1 binds circulating leukocytes through LFA-1 (CD11a, CD18), a member of the β_2 integrin family (Marlin & Springer, 1987). ICAM-1 is a member of the immunoglobin gene superfamily containing five immunoglobulin domains (Simmons *et al.*, 1988; Staunton *et al.*, 1988; Tomassini *et al.*, 1989). Expression of ICAM-1 is inducible by a number of cytokines including IL-1, TNF-α and IFN-γ (Rothlein *et al.*, 1988; Stoolman, 1989; Osborn, 1990; Springer, 1990). The broad tissue distribution of ICAM-1 suggests that it is not only involved in the emigration of leukocytes out of the vasculature, but may play a more extensive role in immune responses. Additional roles suggested for ICAM-1 include localization of leukocytes to the area of inflammation in extravascular spaces, enhancement of the recognition of antigen presenting cells by T lymphocytes, formation of lymphocyte germinal centers, enhancement of natural killer cell response and differentiation of thymocytes (Rothlein *et al.*, 1986; Dustin *et al.*, 1986 & 1988; Makgoba *et al.*, 1988; Altmann *et al.*, 1989; Boyd, 1989; Robertson *et al.*, 1990; Springer, 1990). In addition ICAM-1 is the receptor for over 90% of the rhinovirus serotypes (Staunton *et al.*, 1989; Tomassini *et al.*, 1989).

***In vitro* inhibition of ICAM-1 expression by antisense oligonucleotides**

During the initial evaluation of a series of phosphorothioate oligonucleotides targeted to specific sites within the ICAM-1 mRNA it was found that the cationic lipid,

of ISIS 1082 in an aqueous
resulted in curative activity
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Table II Leukocyte adhesion molecules

Endothelial CAM	*Expressed on other cells*	*Gene family*	*Induction kinetics*	*Leukocyte ligand*	*Type of leukocyte bound*
ICAM-1	Keratinocytes, fibroblasts, leukocytes, etc.	Immunoglobulin	4 h to 72 h	LFA-1, MAC-1	Lymphocytes, monocytes, granulocytes
ICAM-2	Activated lymphocytes	Immunoglobulin	Constitutively	LFA-1	Lymphocytes, monocytes, granulocytes
VCAM-1	No	Immunoglobulin	4 h to 72 h	VLA4	Lymphocytes, monocytes
ELAM-1	No	LEC-CAM	2 h to 18 h	Carbohydrate	Granulocytes monocytes, memory T cells
GMP-140	Platelets	LEC-CAM	5 min to 2 h	Carbohydrate	Granulocytes, monocytes

DOTMA markedly enhanced the activity of the antisense oligonucleotides used in this study. DOTMA was originally described as a vehicle for transfection of DNA into cells (Felgner *et al.*, 1987). Cationic lipid delivery methods differ from normal liposomal delivery methods, in that the DNA or oligonucleotide is not encapsulated within the liposome, but rather is associated with the surface of the liposome through ionic interactions. Preliminary data in certain cell lines indicate that DOTMA enhances cell association of oligonucleotides at least 10-fold and markedly changes the intracellular distribution of the oligonucleotide, with apparently less oligonucleotide being concentrated in endosomes or lysosomes and more found in the nucleus (Chiang *et al.*, 1991; Bennett *et al.*, in preparation). Therefore, in some cells DOTMA will enhance oligonucleotide entry into the cytoplasm of cells similar to direct microinjection. The use of DOTMA has the advantage over microinjection experiments in that oligonucleotides can be introduced into large number of cells allowing biochemical analysis to be performed. In addition, it was determined that DOTMA had no effect on the expression of ICAM-1 when used at concentrations that maximized oligonucleotide uptake and activity (Chiang *et al.*, 1991). The use of DOTMA in these experiments allowed us to determine which regions on the ICAM-1 mRNA serve as the best target sites for antisense oligonucleotides and determined the mechanism by which antisense oligonucleotides inhibit ICAM-1 expression. To our knowledge this is the first report demonstrating that cationic lipids enhance antisense oligonucleotide activity in mammalian cells.

Using DOTMA as a formulation medium we have demonstrated that antisense oligonucleotides which target human ICAM-1 mRNA inhibit the expression of ICAM-1 in two cell culture systems HUVEC and a human lung carcinoma, A549 (Chiang *et al.*, 1991). Screening antisense oligonucleotides which target a number of sites on the ICAM-1 mRNA revealed that two sites were especially sensitive to inhibition with antisense oligonucleotides; the AUG translation initiation codon and specific sequences in the 3'-untranslated region. Data from these studies suggest that hybridization affinity is important for antisense oligonucleotides, as truncated versions of active oligonucleotides (<20-mers) exhibit decreased activity, however, hybridization affinity is not sufficient to ensure antisense activity. Therefore, target site selection is also an important parameter to consider when designing antisense oligonucleotides.

The most active ICAM-1 antisense oligonucleotide targets the 3'-untranslated region of the ICAM-1 mRNA. ISIS 1939 hybridizes to the ICAM-1 mRNA, nearly 300 bases 3'- to the translation termination site, therefore it should not directly affect translation of the protein. This oligonucleotide was shown to inhibit the expression of ICAM-1 in endothelial cells as measured by ELISA using a monoclonal antibody to ICAM-1 (Figure 2). Under equivalent experimental conditions treatment of endothelial cells with ISIS 1939 blocked the adhesion of HL60 cells. Thus the blockade of ICAM-1 expression was coincident with the loss of functional activity of the protein. Oligonucleotides which hybridized to other sequences in the 3'-untranslated region of ICAM-1 mRNA were not as effective as ISIS 1939 (Figure 2). Therefore, the effects of ISIS 1939 are unique to the target site to which it hybridizes.

ICAM-1 mRNA contains three repeats of a consensus sequence, AUUUA, thought to be involved in destabilization of mRNA (Caput *et al.*, 1986; Shaw & Kamen, 1986; Brawerman, 1989). An oligonucleotide that targets those sequences was shown to exhibit weak activity. However, ISIS 1939 targets an area approximately 200 bases 5'- to the AUUUA sequences. The region targeted by ISIS 1939 is predicted to be a stable stem loop structure which when bound would disrupt the structure. Analysis of steady state mRNA levels from oligonucleotide treated cells revealed that ISIS 1939

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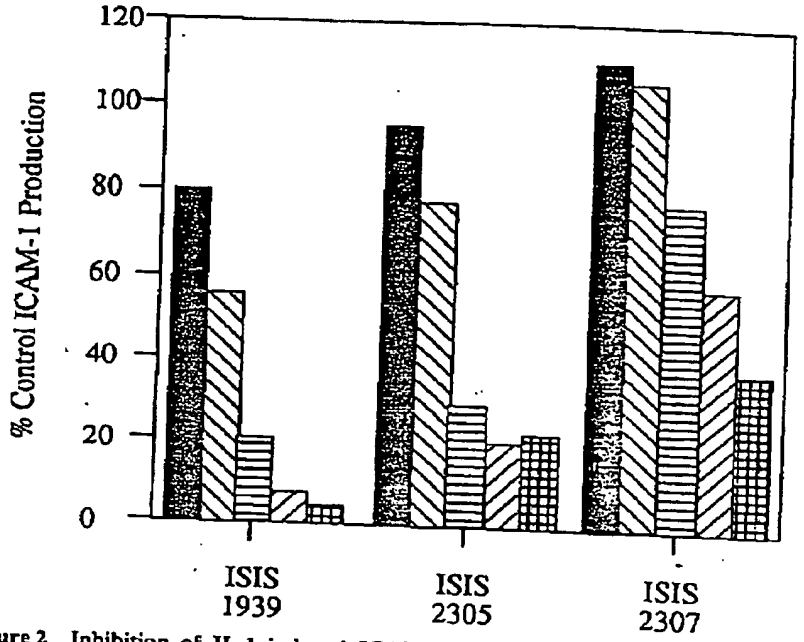

Figure 2 Inhibition of IL-1 induced ICAM-1 expression in A549 cells with antisense oligonucleotides which hybridize to the 3′-untranslated region of ICAM-1 mRNA. Cells were treated with phosphorothioate oligonucleotides (20 mers) at concentrations of ■ 0.1 μM ▨ 0.3 μM ▤ 0.5 μM ▨ 0.7 μM ▦ 1.0 μM in the presence of DOTMA. ICAM-1 expression was measured by ELISA using an ICAM-1 monoclonal antibody 84H10

specifically reduced the quantity of ICAM-1 mRNA per cell. The reduction of ICAM-1 mRNA was not due to decreased transcription of the ICAM-1 gene as analysed by nuclear run-off reactions. Therefore, ISIS 1939 must destabilize the ICAM-1 mRNA either by an RNase H dependent mechanism and/or by modulating natural processes which help to stabilize the ICAM-1 mRNA.

Oligonucleotides targeted to certain other specific sites within ICAM-1 mRNA were found to be potent inhibitors of ICAM-1 protein expression and cell adhesion. These oligonucleotides were targeted to sequences within the 5′ untranslated region and the translation initiation region. The oligonucleotide targeted to the translation initiation region did not cause a reduction in the steady state level of ICAM-1 mRNA; unlike that found with ISIS 1939. Taken together these data suggest that different oligonucleotides targeted to different sites on an RNA may inhibit the production of a protein by different mechanisms.

Summary

The notion of using antisense oligonucleotides as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and

chemical properties that govern the structure of nucleic acids. The practical evidence that antisense oligonucleotides can be drugs is a result of the work of a number of laboratories, including those cited in this review.

Key to the continued progress in the field of antisense therapeutics is the realization that oligonucleotides and their RNA targets work via the same principles of pharmacology that govern the actions of all other classes of drugs. Considering the properties of drugs that define their pharmacologic value, such as ligand-receptor binding affinity and fidelity and realizing the intrinsic properties of oligonucleotides, it is very clear that these compounds have enormous potential value in treating human diseases.

During the next few years a number of oligonucleotide compounds will enter into clinical trials. These first generation antisense drugs (e.g. phosphorothioates) will encounter many of the same issues and hurdles that confront all novel pharmaceutical agents; large-scale process development, adequate methods and tools to define clinical pharmacokinetics and metabolism, etc. Another important component of this process is the continued examination and definition of the molecular pharmacodynamics and pharmacokinetics of these drugs. We need to better understand how the structure and function of RNA defines the sensitivity of specific target sites to antisense oligonucleotides, the precise role of RNase H and other intracellular enzymes and proteins in the mechanism of action in oligonucleotides, the process by which oligonucleotides penetrate cellular membranes and distribute in cells, the non-sequence specific interactions that oligonucleotides can engage in both in and out of cells, and the metabolic pathways (both nuclease and non-nuclease) and metabolites that are likely to play a role in the metabolism of antisense drugs. The combination of this molecular, cellular, and clinical information will allow us to better determine the specific molecular targets and diseases that can be successfully treated with the first generation of antisense drugs. As important, it will define the biology, chemistry, and pharmacology of second and third generation antisense drugs.

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